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TITLE OF THE INVENTION

GLOBAL REGULATORS OF BACTERIAL PATHOGENIC GENES; BACTERIAL AUTOINDUCER INACTIVATION PROTEIN, AS TARGETS FOR ENGINEERING DISEASE RESISTANCE

CROSS-REFERENCES TO RELATED APPLICATIONS

Not applicable.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to global regulators of bacterial pathogenic genes, and their use to confer disease resistance.

2. Description of the Related Art

A bibliography follows at the end of the Detailed Description of the Invention. The listed references are all incorporated herein by reference.

Cell-to-cell communication via small signal molecules is not only of vital importance to multi-celled living organisms such as animals and plants, it also plays important roles in the functional co-ordination among family members of single-celled organisms like bacteria. Rapid progress over the last few years has clearly established that N-acyl-homoserine lactones, known as autoinducers

(AIs), are widely conserved signal molecules in Gram-negative bacteria. AIs were first found in marine bacteria *Vibrio* species in regulation of bioluminescence (Eberhard, *et al.*, 1981; Cao and Meighen, 1989). In recent years, AIs have been identified in a wide range of Gram-negative bacteria. It has been found that AIs are involved in the regulation of a range of biological functions, including Ti plasmid conjugal transfer in *Agrobacterium tumefaciens* (Zhang, *et al.*, 1993), induction of virulence genes in *Erwinia carotovora*, *Pseudomonas aeruginosa*, *Erwinia stewartii*, *Xenorhabdus nematophilus*, *Erwinia chrysanthemi*, *Pseudomonas solanacerum*, and *Xanthomonas campestris* (Jones, *et al.*, 1993; Passador, *et al.*, 1993; Pirhonen, *et al.*, 1993; Pearson, *et al.*, 1994; Beck von Bodman and Farrand, 1995; Barber, *et al.*, 1997; Clough, *et al.*, 1997; Costa and Loper, 1997; Dunphy, *et al.*, 1997; Nasser, *et al.*, 1998), regulation of antibiotics production in *Pseudomonas aureofaciens* and *Erwinia carotovora* (Pierson, *et al.*, 1994; Costa and Loper, 1997), regulation of swarming motility in *Serratia liquifaciens* (Eberl, *et al.*, 1996), and biofilm formation in *Pseudomonas fluorescens* and *P. aeruginosa* (Allison, *et al.*, 1998; Davies, *et al.*, 1998). Many more bacterial species are known to produce AIs but the biological functions related have not been established yet (Bassler, *et al.*, 1997; Dumenyo, *et al.*, 1998; Cha, *et al.*, 1998).

Different bacterial species could produce different AIs. All AI derivatives share identical homoserine lactone moieties but can differ in the length and the structure of their acyl groups. The key

components in AI-mediated gene regulation systems are LuxI and LuxR type proteins. It has been established now that LuxI-type protein serves as an autoinducer synthase that utilizes acyl-ACPs and AdoMet (S-adenosylmethionine) as substrates (More, *et al.*, 1996; Schaefer, *et al.*, 1996). LuxR-type protein is proposed to be both a receptor for AIs and a AI-dependent transcriptional regulator that binds DNA immediately upstream of the *lux* promoter (Meighen, 1994; Sitnikov, *et al.*, 1995). A 20-nucleotide inverted repeat has been identified which is centered 44 nucleotides upstream of the transcription start site of the luminescence operon. This sequence called *lux* box is required for transcriptional activation by LuxR and is probably the LuxR binding site (Fuqua, *et al.*, 1994). Similar 18-bp *tra* boxes are found upstream of at least three TraR-regulated promoters, and disruption of these elements abolishes transcriptional activation by TraR (Fuqua and Winans, 1996a).

LuxR-type proteins appear to be composed of two modules (Choi and Greenberg, 1991; Hanzelka and Greenberg, 1995). Their carboxyl terminal regions contain a conserved short sequence of 19-amino acid, putative probe-type helix-turn-helix motif, predicted to be involved in binding to target promoters. A general mechanism of activation has been proposed by which the N-terminal domain of LuxR-type protein acts negatively to prevent an interaction between its C-terminal domain and the target DNA binding sites. This inhibition can be relieved by the action of an autoinducer ligand. A strong piece of evidence is that deletion of the N-terminal domain of LuxR results in constitutively active alleles of *lux*R, whereas larger

deletions that remove part of the predicted DNA binding domain abolish transcriptional activation (Choi and Greenberg, 1991). However, other members might use different mechanisms. Recent genetic studies indicate that EsaR and ExpR are likely to be repressors of their target genes rather than activators. Expression of the genes that are repressed by EsaR and ExpR is increased by autoinducers (Beckvon Bodman and Farrand 1995; Throup, *et al.* 1995). It appears that binding of these proteins to their target sites in promoter region causes repression, therefore autoinducer ligands may act to reduce binding affinity.

Evidence that the autoinducer binding site resides in the amino terminal domain of the LuxR protein has been presented (Hanzelka and Greenberg, 1995). LuxR alleles that have mutated amino terminal region require higher level of this signal that does the wild type, indicating this region required for ligand interaction (Slock, *et al.*, 1990; Shadel, *et al.*, 1990). This region (aa 79-127) and a region within the DNA-binding domain (aa 180-230) show a higher degree of conservation among LuxR and its homologs (ca 50% identity) than other parts of these polypeptides. However, the proposed protein-ligand interaction between LuxR and autoinducer has not been proved yet. Analysis of merodiploid *E. coli* strains containing wild-type and mutant LuxR alleles suggested that LuxR functions as a homomultimer and that a region required for multimerization resides within amino acid residues 116 and 161 (Choi and Greenberg, 1992).

BRIEF SUMMARY OF THE INVENTION

In one aspect, the present invention relates to an isolated nucleic acid molecule encoding a bacterial autoinducer inactivation protein.

In another aspect, the present invention relates to a expression vector which comprises a nucleic acid molecule encoding a bacterial autoinducer inactivation protein, wherein the expression vector propogates in a procaryotic or eucaryotic cell.

In yet another aspect, the present invention relates to a cell of a procaryote or eucaryote transformed or transfected with the expression vector of the present invention.

In yet another aspect, the present invention relates to an isolated protein which has bacterial autoinduction inactivation activity, where the protein comprises the amino acid sequence of SEQ ID NO: 2.

In yet another aspect, the present invention relates to a method for increasing disease resistance in a plant or animal, which method comprises introducing into a cell of such plant or animal a nucleic acid sequence which encodes a bacterial autoinducer inactivation protein in a manner which allows said cell to express said nucleic acid sequence.

In yet another aspect, the present invention relates to a method of preventing or reducing bacterial damage to a plant or animal, which method comprises administering to a plant or animal in need of such prevention or reduction an effective amount of a bacterial autoinducer inactivation protein.

In yet another aspect, the present invention relates to a composition for reducing bacterial damage to a plant or animal, which comprises:

a) an effective amount of a bacterial autoinducer inactivation protein; and

b) a suitable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the time course of AIs inactivation by cell extracts from Bacillus sp. strain 240BI. Cell extracts in 0.2 M phosphate buffer (pH 7.0) containing 100 ug total protein were added to the same buffer containing OHHL in a final concentration of 20 uM. The reaction was conducted in a 1.5 ml Eppendorf centrifuge tube in a final volume of 200 microliters and incubated at 28°C. Same concentration of OHHL in the phosphate buffer was used as control. Samples were taken at 10-min interval till 60 min and the reaction was stopped by boiling for 3 min. The samples were centrifuged for 5 min in a bench top centrifuge at the top speed and then assayed for AIs activity as described (Zhang, 1993). Blue colony indicates the presence of AI that activates the lacZ reporter gene, and white colony indicates absence of AI. Rows from left to right: 1, OHHL control without protein extract; 2 - 7, samples after 10, 20, 30, 40, 50, 60 min enzyme reaction.

Figure 2 shows the estimation of molecular mass of AIs inactivation enzyme. A 600 µl aliquot of cell extracts was added to the Centricon 30 (Amicon) and was centrifuged at a speed of 5000 x g for 30 min at 4°C. Passing fraction (550 microliters) and un-passing fraction (50 microliters) were topped up separately to a final volume of 600 microliters by adding 0.2 M phosphate buffer (pH 7.0). For bioassay, different

amounts of protein samples were added to the tubes containing OHHL in a final concentration of 20 ,uM. From row 1 to 6, protein samples added were 2, 4, 6, 8, 10 and 0 ,ul and the final reaction volume was 20 microliters for each reaction. Plate A: Passing fraction, Plate B: un-passing fraction.

Figure 3 shows the cloning and deletion analysis of Bacillus SP. strain 240B1 AI inactivation region. Cosmid clone E7-R3 contains the 4.3-kb EcoRI fragment identified by restriction analysis of overlapping cosmid clones. For deletion analysis, the same fragment was cloned into cloning vector pGEM-7Zf(+) for generation of clone E7-7. The deletion subclones were produced by restriction enzyme digestion and Dnase I treatment from the clone E7-7. The location and direction of Ptac promoters in the cosmid and in the pGEM-7Zf(+) clone are indicated by arrows. AI inactivation activity of the clones is shown in the second column: +, with AI inactivation activity; -, without Al inactivation activity. Restriction enzymes: E, EcoRI; H, HindIII; Ev, EcoRV; St, StyI. The location and direction of transcription of the aiiA ORF is indicated by an open arrow.

Figure 4A shows the nucleotide sequence of the aiiA gene [SEQ ID NO:1]. The potential ribosome binding sequence and -10 promoter element are underlined and double underlined respectively. The coding portion starts at base 1. The putative factor-independent termination site is labeled by a thick underline. Figure 4B shows the predicted amino acid sequence of the aiiA gene product [SEQ ID NO:2].

A short peptide sequence similar to the aspartyl protease active site consensus motif is underlined.

Figure 5 shows the best match of amino acids sequence of *aiiA* gene product (AiiA) to the concensus aspartyl proteases active site motif (Asp). Symbol: X, any amino acid. A vertical line indicates perfect match.

Figure 6 shows the bioassay for AIs inactivation activities in *Bacillus sp.* strain 240B1, E.coli clones and AIs production activity in *Erwinia carotovora* strains. Row 1, OHHL control; row 2, *Bacillus* sp. strain 240BI; row 3, *E. coli* DH5α; row 4, *E. coli* DH5α (pE7-R3); row 5, *E. coli* DH5α (pF41); row 6, *Erw. carotovora* SCG1(pE7R3); row 7, *Erw. carotovora* SCG1(pLAFR3); row 8, *Erw. carotovora* SCG1. In the bioassay, OHHL was added to a final concentration of 20 µM to the samples from lines 1 to 5. No exogenous AIs were added to the samples from rows 6 to 8.

Figure 7 shows the effect of *aiiA* gene expression in *Erw. carotovora* on pathogenicity in (A), potato; (B), eggplant; (C), Chinese cabbage; (D), carrot; and (E), celery. Top: plant tissues were inoculated with *Erw. carotovora* SCG1. Bottom: plant tissues were inoculated with *Erw. carotovora* SCG1 (pE7-R3). The actively growing bacteria were centrifuged for 1 min at 3000 x g, resuspended with YEB liquid medium to OD600 = 1.3 (2 x 10^9 cfu/ml) which was designed as 10^0 inoculum. The 10^0 inoculum was diluted 5 and 10 times respectively to prepare $10^{-1/2}$ and 10^{-1} dilutions. The Plant tissues were inoculated by adding a 4-µl volume of bacteria

inoculum to the freshly cut surface or a wounding site punched by a pipette tip. The inoculum concentration from the left to the right plate: 10^0; $10^{-1/2}$; and 10^{-1}. The inoculated plant tissues were placed in plastic plates and incubated at 28°C. The photograph was taken 48 h after inoculation.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery that the SEQ ID NO:2 protein has the effect of reducing or eliminating the activity of bacterial autoinducers (AIs). Consequently, the protein, and any nucleic acid that encodes the protein, may be used in a variety of situations where it is desired to reduce or eliminate the effect of such bacteria.

In one preferred aspect, the present invention provides a nucleic acid molecule which is selected from the group consisting of:

a) a nucleic acid having the sequence of SEQ ID NO:1;

b) a nucleic acid encoding the amino acid sequence of SEQ ID NO:2; and

c) a nucleic acid that hybridizes to a) or b) above, wherein a positive hybridization signal is observed after washing with 1 X SSC and 0.1% SDS at 55°C for one hour. The nucleic acid optionally further comprises a signal peptide coding region of any sequence.

The nucleic acid sequence may be used to confer bacterial resistance in plants or animals. A nucleic acid that encodes a bacterial autoinducer inactivation protein can be introduced into a cell such that the

inactivation protein is expressed by the plant or animal.

The nucleic acid sequence may be used to confer resistance to diseases where the expression of pathogenic genes are regulated by autoinducers, such as the diseases caused by *Pseudomonas aeruginosa*, *Erwinia stewartii*, *Xenorhabdus nematophilus*, *Erwinia chrysanthemi*, *Pseudomonas solanacerum*, and *Xanthomonas campestris* (Passador, *et al.*, 1993; Pirhonen, *et al.*, 1993; Pearson, *et al.*, 1994; Beck von Bodman and Farrand, 1995; Barber, *et al.*, 1997; Clough, *et al.*, 1997; Costa and Loper, 1997; Dunphy, *et al.*, 1997; Nasser, *et al.*, 1998). Preferably, in the agricultural setting, the sequence may be used to confer soft rot disease resistance in susceptible plants, such as potato, eggplant, Chinese cabbage, carrot and celery.

The sequence may be introduced into plant or animal cells by well-known methods. Methods for the transformation or transfection of eukaryotic cells with exogenous nucleic acid sequences include transfection, projectile bombardment, electroporation or infection by *Agrobacterium tumefaciens*. These methods are likewise familiar to the person skilled in the area of molecular biology and biotechnology and need not be explained here in detail. As pathogenic bacteria cells are confined to the intercellular area of plant tissues, it is desirable to target the AiiA protein into the intercellular spaces. Such may be accomplished by fusing a secretion signal peptide to the AiiA protein (Sato, *et al.*, 1995; Firek, *et al.*, 1993; Conrad and Fiedler, 1998; Borisjuk, *et al.*, 1999). Alternatively, a plant membrane attachment motif can be incorporated into the peptide sequence of AiiA for anchoring the

AiiA enzyme in the outer surface of plant cell membrane.

The present invention provides a new strategy for engineering resistance to diseases. In particular, this strategy targets N-acyl homoserine lactone autoinducers that induce expression of pathogenic genes of many bacterial pathogens at a threshold concentration. This strategy is applicable to all plant, animal or mammal diseases where the expression of pathogenic genes of the bacterial pathogens is inducible by N-acyl homoserine lactone autoinducers.

The present invention also contemplates usage of a bacterial autoinducer inactivation protein directly to treat or prevent bacterial damage. For example, the protein may be applied directly to plants in need of such treatment or prevention. In a preferred embodiment, the protein is applied in the form of a composition which comprises an effective amount of the protein and a suitable carrier. The composition may have a wide variety of forms, including solutions, powders, emulsions, dispersions, pastes, aerosols, *etc.*

The bacterial autoinducer inactivation protein may also be used to treat bacterial infections in animals, including humans. In that application, an effective amount of the active ingredient is administered to an animal in need of such treatment.

For therapeutic treatment, the active ingredient may be formulated into a pharmaceutical composition, which may include, in addition to an effective amount of the active ingredient, pharmaceutically acceptable carriers, diluents, buffers, preservatives, surface active agents, and the like. Compositions may also

include one or more other active ingredients if necessary or desirable.

The pharmaceutical compositions of the present invention may be administered in a number of ways as will be apparent to one of ordinary skill in the art. Administration may be done topically, orally, by inhalation, or parenterally, for example.

Topical formulations may include ointments, lotions, creams, gels, drops, suppositories,sprays, liquids and powders. Oral formulations include powders, granules, suspensions or solution in water or non-aqueous media, capsules or tablets, for example. Thickeners, flavorings, diluents, emulsifiers, dispersing aids or binders may be used as needed.

Parenteral formulations may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives.

The dose regimen will depend on a number of factors which may readily be determined, such as severity and responsiveness of the condition to be treated.

Aspects of the invention will now be illustrated with reference to the following non-limiting examples.

EXAMPLE 1

Bacterial isolate 240B1 was isolated from soil suspension based on its function for inactivation of N-ß-oxo-hexanoyl-L-homoserine lactone (OHHL) and N-ß-oxooctanoyl-L-homoserine lactone (OOHL) and N-ß-oxodecanoyl-L-homoserine lactone (ODHL) (Zhang, *et al.*, 1993). Unless otherwise stated, OHHL was used for routine bioassay. *Erwinia carotovora* strain SCG1 was isolated from Chinese cabbage leaf showing soft rot

symptoms. It has been confirmed that strain SCG1 produces AIs and elicits soft rot disease in potato and Chinese cabbage. *Escherichia coil* strain DH5α was used as a host for DNA cloning and subcloning. *Agrobacterium tumefaciens* strain NT1 (*traR; tra::lacZ749*) was used as an indicator in bioassay for AI activity (Piper, *et al.*, 1993). *E. coli* strain was cultured in Luria-Bertani (LB) medium at 37°C and other strains were cultured in LB (Miller, 1972) or YEB medium (per liter contains: casein hydrolysate 10 g, yeast extract 5 g, NaCl 10 g, sucrose 5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, agar 15 g, pH 7.2) at 28 °C. The minimal salts medium with mannitol and $(NH4)_2SO_4$ as carbon and nitrogen sources was used for bioassay of OHHL (Petit and Tempe, 1978). Appropriate antibiotics were added as indicated at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 20 μg/ml and kanamycin, 50 μg/ml.

Bioassay of AIs activity

The qualitative and quantitative bioassay methods for determination of AIs activity has been described previously (Zhang, 1993). For determination of the AIs production ability of wild-type and genetically modified *Erwinia* strains, the same bioassay procedure was used except that no OHHL was added into the bacterial culture.

Cloning and sequencing of the AiiA gene

Genomic DNA from 240B1 was digested partially with *Eco*RI. DNA fragments were ligated to the dephosphorylated *Eco*RI site of cosmid vector pLAFR3 (Staskawicz, *et al.*, 1987). Ligated DNA was packaged

with Gigapack III XL Packaging Extract (Stratagene) and transfected into *E. coil* DH5α. Cosmid clones with OHHL inactivation activity were identified by using the bioassay method described above. Subcloning into sequencing vector pGEM-7Zf(+) was carried out by routine techniques (Sambrook, *et al.*, 1989). Deletion analysis was carried out by using DnaseI method as described by Lin, *et al.* (1985). The sequencing was performed on both strands using the ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). Nucleic acid sequence data and deduced amino acid sequences were analyzed with a DNASTAR™ sequence analysis software package (DNASTAR Inc.) and database searches were performed using the BLASTA search algorithm (Altschul, *et al.*, 1990).

Genetic modification of Erwinia strain SCG1

The E7-R3 plasmid, carrying the *aiiA* gene in the cosmid vector pLAFR3, was transferred into *Erwinia* stain SCG1 by triparental mating with the helper strain RK2013 (Ditta, *et al.*, 1980). Transconjugants were selected on the plates containing minimal medium with tetracycline and confirmed by PCR with primers specific to the *aiiA* gene.

Virulence tests

The virulence of wild-type *Erw. carolovora* strain SCG 1 and the aiiA gene transformant SCG1(E7-R3) was evaluated by inoculation. Four µl of early stationary phase bacterial suspension (containing ~2 x 10^9 cell/ml) or diluted bacteria was added to the cut surfaces or wounding sites of plant tissues. The inoculated plant

tissues were incubated in a Petri dish at 28°C overnight. The severity of soft rot was examined 48 hours after incubation.

Results

Screening of bacteria that inactivate AIs

Bacterial isolates from plant and soil samples were screened for enzymatic inactivation of AIs. A bacterial isolate 240B1, which showed a strong ability to eliminate AIs activity, was selected for further study. The total protein extracts from isolate 240B1 eliminated AIs activity completely during one-hour incubation (Fig. 1), and the capacity of the protein extract to inactivate AIs was abolished by treatment with proteinase K for 1 hour or boiling for 5 min. These observations indicate enzymatic inactivation of AIs by bacterial isolate 240B1. The isolate was taxonomically characterized as *Bacillus* sp., because of the following characteristics: Gram-positive, rod-shaped, catalase positive, facultatively anaerobic, and 16 rRNA sequence homology to that of other *Bacillus* bacteria (data not shown).

The molecular mass of the enzyme for AIs inactivation appears to be larger than 30 kDa. Its activity was lost after passing the protein extract through Centricon 30 (Amicon) but the activity was recovered in the re-suspended fraction that failed to pass the Centricon 30 (Fig. 2).

Cloning and localization of AIs inactivation region

To identify the gene encoding AIs inactivation, a cosmid library was constructed with the genomic DNA of *Listera* sp. strain 240B1. Twelve hundred clones were

screened for AIs inactivation activity. Three clones showing AIs inactivating function were identified. Restriction analysis showed that the 3 clones shared one common band of 4.3-kb generated by *Eco*RI digestion. The bioassay with the subclone E7-7 containing this 4.3-kb *Eco*RI fragment confirmed that this fragment encodes AIs inactivation function (Fig. 3). To identify the minimum size and the location of the AIs inactivation gene (*aiiA*), a serial of deletion clones was generated by deletion from both ends of this 4.3-kb fragment with the DNaseI method (Lin, *et al.*, 1985). The results indicated that the *aiiA* gene is contained in a 1.2 Kb fragment in clone F41 (Fig. 3).

***AiiA* gene encodes a novel protein**

The 1.2-kb DNA insert in clone F41 was completely sequenced from both strands. The nucleotide sequence of aiiA and the predicted amino acid sequence are shown in Fig. 4. The complete sequence of the DNA insert contains 1,222 base pairs and there are 4 potential in-frame open reading frames (ORF) starting from nucleotide position of 1, 42, 156 and 228 respectively (Fig. 4). Deletion analysis indicated that only the longest ORF encodes AIs inactivation function, because the clone R34, in which the 48 bp promoter region and nucleotides from 1 to 13 in the longest ORF were deleted, lost AI inactivation function completely, although the remaining DNA insert was placed under the control of a functional Ptac promoter (Fig. 3). This is confirmed by fusing the longest ORF to the glutathione S-transferase gene in the same ORF and testing for AI inactivation activity of the purified fusion protein (data not shown). This ORF contains 750

bp nucleotide and encodes a protein of 250 amino acids, with a predicted molecular mass of 28,036 daltons and an isoelectric point at 4.7, because of 19 strongly basic and 39 strongly acidic amino acids residues. The putative initiation codon is preceded at a spacing of 7 bp by a potential ribosome-binding sequence (AAGGTGG) which is complementary to the 3' end of the *E. coli* 16S rRNA. The best sequence match (TATTGT) to the consensus -10 promoter element (TATAAT) occurs 35 bp upstream of the initiation codon. A TCTT box following a T-rich region resembling the potential factor-independent termination site is found downstream of the termination codon (Brendel, 1986). The total GC content of the *aiiA* gene is 37% and GC content in the third position of the codon is 27.2%.

Database searches showed that the aiiA gene has no significant similarity to known sequences in the major databases (GenBank, European Molecular Biology Laboratory, Protein Information Resource, and Swiss-Prot) by FASTA and BLAST analysis at either nucleotide or peptide sequence level, suggesting that *AiiA* is a novel protein. Consensus protein motif search using the Genetics Computer Group (Madison, WI) MOTIF program showed that a short peptide sequence, "ILVDTGMPESAV" from position 47 to 58 in AiiA, is similar but not identical to the aspartyl protease active site signature pattern (Rawlings and Barrett, 1995) (Fig. 5).

Expression of aiiA gene in Erwinia carotovora decreases AIs releasing and attenuates virulence

The cosmid clone E7-R3 was transferred into *Erwinia carotovora* strain SCG1 by triparental mating. The pLAFR3 vector has been safely maintained in *Erwinia*

carotovora without selection pressure. The bioassay showed that the AIs released by *Erwinia carotovora* (E7-R3) was significantly reduced (Fig. 6, lane 6), while the presence of the cosmid vector pLAFR3 alone in *Erwinia carotovora* did not affect AIs production (Fig. 6, lanes 7). Data suggest that the most of AIs produced by *Erwinia carotovora* strain SCG1 was inactivated by *aiiA* gene product.

The *Erwinia carotovora* SCG1(E7-R3) that expresses AiiA protein failed to or caused only minor soft rot disease symptom in potato, eggplant, Chinese cabbage, carrot and celery, while its parental strain caused severe symptoms (Fig. 7A, B, C, D, E). To prevent experimental errors due to genetic variations, four colonies from *Erwinia carotovora* strain SCG1 and its *aiiA* gene transformants respectively, were randomly selected for testing AIs production and virulence on potato. Similar results were obtained in both experiments. The *Erwinia carotovora* strain SCGI (pLAFR3) that contains the cosmid vector only caused the same level of disease severity as its parental strain *Erwinia carotovora* strain SCGI (Fig. 7F).

Discussion

Bacterial isolate 240B I, which was identified as *Bacillus* sp., produces an enzyme that can effectively inactivate the three AIs tested, *i.e.,* N-ß-oxo-hexanoyl-L-homoserine lactone, N-ß-oxo-octanoyl-L-homoserine lactone and N-ß-oxo-decanoyl-L-homoserine lactone. The gene (*aiiA*) encoding the AI inactivation enzyme has been cloned and fully sequenced. Expression of the *aiiA* gene in transformed *E. coli* and pathogenic bacteria *Erwinia carolovora* confers ability for AI

inactivation and significantly reduces the AIs release from *Erwinia carolovora*. To our knowledge, it is the first protein identified capable of enzymatic inactivation of N-acyl-homoserine lactones, the autoinducers for global gene regulation in a diverse of bacteria species.

The AiiA is a novel protein. There is no significant homology to known proteins in major databases. It shares similarities to the consensus pattern of the aspartyl proteases active site (Rawlings and Barret, 1995). Aspartyl proteases, also known as acid proteases, are widely distributed in vertebrates, fungi, plants, retroviruses and some plant viruses. The aspartyl proteases from most retroviruses and some plant viruses are homodimers. The molecular mass of AiiA protein is about 28 kDa but it failed to pass a molecular sieve with a cut off size of 30 kDa, indicating a possibility that AiiA protein exists as a homodimer or homomultimer under the natural conditions. However, there is also a possibility that AiiA monomer has an irregular three-dimensional structure, which hinders it passing through the molecular sieve. Aspartyl proteases are endopeptidases and hydrolyses amide linkages of proteins. Crystallographic study has shown that the enzyme of the aspartyl protease family are bilobed molecules with the active-site cleft located between the lobes, and each lobe contributing one of the pair of aspartic acid residues that is responsible for the catalytic activity (Sielecki et al., 1991).

Erwinia carotovora is a plant pathogen that produces and secretes exoenzymes that act as virulence determinants for soft rot diseases of various plants

including potato, cabbages, tomato, chili, carrot, celery, onion, and lettuce (Kotoujansky, 1987).

Mutants that were defective in the producing *N*-3-(oxohexanoyl)-L-homoserine lactone were also defective in systhesis of the pectinase, cellulase and protease exoemzymes. These mutants failed to induce soft rot disease in potato tubers (Jones, *et al.*, 1993). It was found that the *expI* gene, which is homologous to *luxI* gene of *Vibrio fischeri*, encodes autoinducer production in *Erwinia carotovora*. The *expI* mutant was avirulent when it was inoculated to tobacco leaf but the virulence was restored by external antoinducer addition (Pirhonen, *et al.*, 1993). Obviously, autoinducers are a potential target for genetic engineering of plant soft rot disease resistance. As an interim test and a concept proving approach, the cosmid clone containing the *aiiA* gene was introduced to *Erwinia carotovora* strain SCG1. Expression of the AiiA enzyme in *Erwinia carotovora* significantly reduced the release of autoinducers, and the genetically modified *Erwinia carotovora* that expressed AiiA failed to induce any or induce only minor soft rot disease symptom on all plants tested, including potato, eggplant, Chinese cabbage, carrot and celery. Our results further support the important role of autoinducers in the regulation of expression of virulence genes in *Erwinia carotovora*, and the potential of the *aiiA* gene to confer resistance to soft rot disease and other diseases in which the autoinducers are involved in regulation of pathogenic gene expression.

The present invention provides a new strategy for engineering resistance to diseases. In particular,

this strategy targets N-acyl homoserine lactone autoinducers that induce expression of pathogenic genes of many bacterial pathogens at a threshold concentration. By using the above-memtioned conception-proving approach, the present invention demonstrates that reduction or elimination of autoinducers produced by pathogenic bacteria by an autoinducer inactivation enzyme significantly attenuates pathogenicity of otherwise virulent bacterial pathogen. Because the expression of pathogenic genes in pathogenic bacteria requires a threshold concentration, this AI-inactivation strategy is applicable to all plant, animal or mammal diseases where the expression of pathogenic genes of the bacterial pathogens is inducible by N-acyl homoserine lactone autoinducers.

The *aiiA* gene could also be a useful tool for investigation of the role of AIs in those bacteria where the biological functions regulated by AIs has not been established. In recent years, many more bacteria species have been shown to produce AIs (Bassler, *et al.*, 1997; Dumenyo, *et al.*, 1998; Cha, *et al.*, 1998; Surette, *et al.*, 1999). Some of them are important plant pathogens such as *Psendomonas* and *Xanthomonas* species. The gene knock out approach based on sequence homology could be difficult. The overall levels of sequence similarity of AIs synthase and the related regulatory protein from different genera are rather low, often no higher than 28-35% identity between LuxI-type proteins and 18-25% identity for LuxR-type proteins (Fuqua et al., 1996). However, it is feasible and simple to introduce the *aiiA* gene into these

bacteria to probe the biological functions regulated by AIs.

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